Protocol: 9ASK
Date: 12/2/80

PROTOCOL FOR CELL CULTURE SCREEN, AC GLIOMA, 9ASK

9ASK: Immature AC glioma cells are cultivated in Eagles MEM plus pretested 10% newborn calf serum. Cells are plated, exposed for one hour to inducing agent, followed by one hour of exposure to drug. Percent of reversal of astrocyte formation is estimated.

Origin of tumor line: A polymorphous oligodendroglioma induced by ethylnitrosourea in an SD-JCL rat. Supplied to NCI by Dr. Yokio Sugino.[1]

EXPERIMENT SIZE

General Testing: Duplicate culture dishes for each dose level per material.

Control Group: Eight to ten culture dishes.

TEST SCHEDULE

Day 0: Pipette into each dish 1 x 10(5) AC glioma cells in 2.0 ml of complete growth medium (Eagles **MEM** plus 10% pretested newborn calf serum). Incubate at 37° C in a 5% $C0_2$ atmosphere.

Day 2: Examine culture dishes for satisfactory growth. Remove 0.3 ml of **medium,** add 0.1 ml solution containing 20 mM of the inducing agent (N6,021-Dibutyryl adenosine 3':5' cyclic monophosphoric acid - sodium salt), and incubate for one hour. Examine for astrocyte formation. Add 0.2 ml of drug solution (suspension) and incubate for one hour. Examine for astrocyte reversal, and report results.

EXPECTED CHANGE IN MORPHOLOGY

The AC glioma cells of the cell line which originated from a rat glioma in 1974 have the fibroephithelioid morphology of immature neuroglial cells: i.e., each is an epithelial-like cell with an abundance of cytoplasm.

The morphological change caused by db-cAMP is primarily a shrinkage of the cytoplasm. That is, the cytoplasm has retracted to the center of the cell and the center portion of each cell has rounded up leaving the cytoplasmic processes more obvious than they were (hidden by the cytoplasm) before db-cAMP treatment. Thus, the cell now resembles a mature neuroglial cell (astrocyte or oligodentrocyte).

DOSAGE

To the 1.8 ml of medium inducing agent and cells per dish, add 0.2 ml of vehicle containing 200 mcg of natural product plant extract or synthetic material; or 0.2 ml of natural product fermentation extract. Any material that is cytotoxic must be retested at lower concentrations at three doses at 1-log dose intervals until $\geq$ 51% of cells in both culture dishes do not exhibit cytotoxicity. Confirmation testing is to be conducted at 3 doses at 1-log intervals.

[1] Structure and Functions 3, 103-112, 1978

QUALITY CONTROL

At least 80% of the cells in each control plate must exhibit astrocyte formation after exposure to the inducing agent. Positive control compound is NSC 757, Colchicine. The % reversal must be $\geq$ 51% at at least one dose level from a dose response of 10, 1, and 0.1 mcg/ml.

REPORTING

Mail test and control screening reports (WS 185 and 186) to the data processing contractor.

CRITERIA OF ACTIVITY

Sequential:	Plant and animal extracts, synthetic materials	$\geq$ 51% astrocyte reversal
Confirmation:	Plant and animal extracts, synthetic materials	$\geq$ 51% astrocyte reversal

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9ASK GLIOMA

The basis of this assay is that immature AC glioma cells can be induced by db-cAMP to change to the morphology of mature, differentiated astrocytes, and that treatment with certain anticancer drugs can reverse this astrocyte formation. Distinction between these two types of cells is, therefore, critical. An AC cell (of the cell line which originated from a rat glioma in 1974) is an immature neuroglial cell that resembles an epithelial cell with an abundance of cytoplasm. The astrocyte-like cell (that results from db-cAMP induction of the AC cell) resembles a mature, differentiated neuroglial cell (astrocyte or oligodentrocyte) with very little cytoplasm and with obvious cytoplasmic processes.

AC glioma cells are grown in Eagles MEM plus pretested 10% newborn calf serum on glass or plastic surfaces. Prior to initiating an experiment or to subculture, decant medium, wash cells twice with saline, decant saline and add 0.25% trypsin in saline. Let stand 1-2 minutes. Decant trypsin and place culture without medium in 5% C02 incubator at 37°C for 5-10 minutes to dislodge cell sheet. After 5-10 minutes in the incubator, resuspend cells in medium and count.

A concentration of 1 x 10(5) cells in 2.0 ml of growth medium (Eagles MEM plus 10% newborn calf serum) is plated in 35 mm plastic tissue culture dishes (e.g., Falcon) and incubated for 48 hours at 37°C in an incubator with a 5% C02 atmosphere. Examine cells after 48 hours incubation for confluent monolayer growth of cells of fibroepithelioid morphology with abundant cytoplasm. (Otherwise, do not use.) Remove 0.3 ml of medium from each dish, and add 0.1 ml containing 20 mM of the inducing agent (N6, 021--Dibutyryl adenosine 3':5' cyclic monophosphoric acid, sodium salt). Reincubate for 60 minutes at 37°C in 5% C02 atmosphere, and check culture. For a satisfactory experiment, only dishes displaying at least 80% astrocyte formation can be used.

To the 1.8 ml of growth medium containing the inducing agent, add 0.2 ml of compound in suitable vehicle containing 200 micrograms -- final dish concentration 100 mcg/ml -- and the treated cultures are reincubated for 60 minutes in 5% C02 atmosphere at 37°C. Examine dish and record estimated reversal of astrocyte formation.

Each experiment should contain 8 to 10 control culture dishes that have been treated with the inducing agent with no test agent added, plus 3 to 4 dishes without the inducing agent added. All compounds in a control are to be tested in duplicate dishes. Each experiment is to contain NSC 757 (Colchicine) at final concentrations of 10, 1, and 0.1 mcg/ml as the positive control compound. At least one concentration must result in a 51% or greater reversal of astrocyte formation to be a satisfactory experiment.

Initially, unless otherwise instructed, all compounds with the exception of the positive control are to be tested at a concentration of 100 mcg/ml in duplicate dishes. If there is less than 51% cytotoxicity, and less than 51% reversal of

astrocyte formation, testing is to be considered complete. Cytotoxicity is defined as cell damage, cell loss, floating cells, or holes in cell sheet. Cytotoxicity $\geq$ 51% is to be considered excessive, and no indication of reversal is to be reported. Testing is to be rescheduled at lower concentrations using 1-log intervals and a three-dose dose response until at least one concentration has < 51% cytotoxicity and the degree of reversal of astrocyte formation can be measured. Degree of cytotoxicity is to be coded on input form (WS #186) according to the following scale:

WS#186 Code	% Cytotoxicity
0	0 - 5
1	6 - 15
2	16 - 30
3	31 - 50
4	51 - 70
5	71 - 90
6	$\geq$ 91

Reversal of astrocyte formation is to be coded using the number corresponding to the following degree of inducing agent reversal:

WS#186 Code	% Reversal
0	0 - 5
1	6 - 15
2	16 - 30
3	31 - 50
4	51 - 70
5	71 - 90
6	$\geq$ 91

Any material where a single dish has a cytotoxicity code $\leq$ 3 and a reversal code $\geq$ 6, and the duplicate dish is acceptable, is to be considered an initial active (TSC Code 15). If in the initial testing a single dish has an acceptable % reversal code of $\geq$ 4 and no cytotoxicity, a TSC of 11 will be assigned, and the same sample must be repeated. If reversal is not confirmed in this repeat, testing is complete.

A difference greater than one code number -- e.g., 1 and 3 -- between duplicate dishes mandates a repeat of the test at the same concentration when testing at one dose level. Table 1 provides a synopsis of rules governing initial sample testing and computer assignment of test status codes or suffixes.

If reversal is confirmed (TSC = 15), a B002 sample is to be tested using a three dose, dose response and 1-log intervals. Confirmation of reversal at any dose level with the B002 sample in an acceptable dose response will complete the testing. If there is no reversal of astrocyte formation $\geq$ 51%, the B002 sample is to be retested. Failure to confirm astrocyte reversal with a B002 sample completes the testing of the material. Tables 2 and 3 provide a synopsis of rules governing B002 sample testing. The screener will assign test status code 33 (see Instruction 14).

Final maximum concentrations of acceptable vehicles (coded as designated in Instruction 14) for the astrocytoma testing are: 1% dimethylsulfoxide (DMSO, Veh 11); 1% ethanol (Veh 12); 5% dimethylformamide (Veh 13); 0.1% dioxane (Veh 14); 0.5% 1N HCL (Veh 15); 0.5% 1N NaOH (Veh 16); 10% distilled water (Veh 17); single strength 10% Hanks bal. salt sol. (Veh 18); single (lX) strength Eagles MEM (serumless, Veh 10).

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TABLE 1

9ASK TSC ASSIGNMENT
CRUDE MATERIAL TESTING -- INITIAL SAMPLE

TEST		DISH A		DISH B		COMPUTER ASSIGNMENT	
		Cytotoxicity Code	% Reversal Code	Cytotoxicity Code	% Reversal Code	TSC	Meaning
1st	if	≥ 4	any	dish A value ±1	dish A value ±1	01	Cytotoxic. Repeat at lower dose(s).
	if	≤ 3	≤ 3	dish A value ±1	dish A value ±1	02	Negative complete.
	if:	≤ 3	≥ 4	dish A value ±1	dish A value ±1	11	Repeat same sample.
	if	≤ 3	≥ 6	dish A value ±1	dish A value ±1	15	Ready for B002 sample.
		Whenever cytotoxicity A and cytotoxicity B or % reversal A and % reversal B are more than one code apart (i.e., 3 and 5) code the TSC "34" and repeat.				34	Repeat 2nd
		Following a TSC = 01, a retest is handled as a dose response. If the same concentration that yielded the TSC = 01 from the initial single dose test is noncytotoxic, set the TSC = 34 for all doses in the dose response. Otherwise, work with the concentration that yields the highest dish A code (and is noncytotoxic and otherwise acceptable) or, if two dish A codes are identical, the test with the best combination of codes (dish A and dish B). The entire dose response will be assigned the TSC determined from the best individual test. For a noncytotoxic test, assign the TSC as described for first test. When any individual test in the dose response produces a dish A and dish B set of cytotoxicity or % reversal codes that vary more than a single code, the entire dose respose will be set to TSC = 34.				
		Following a TSC = 02 (any value):				02E	Complete

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TABLE 1
(Continued)

9ASK TSC ASSIGNMENT
CRUDE MATERIAL TESTING -- INITIAL SAMPLE

TEST						
2nd	Following a TSC = 11, evaluate "A" values only after confirming A and B values are within one code and handle as follows:					
	DISH A		DISH B		COMPUTER ASSIGNMENT	
	Cytotoxicity Code	% Reversal Code	Cytoxicity Code	% Reversal Code	TSC	Meaning
if:	≥ 4	any	dish A value ±1	dish A value ±1	03	Cytotoxic. Repeat at lower dose.
if	≤ 3	≤ 3	dish A value ±1	dish A value ±1	06	Negative complete.
if:	≤ 3	≥ 4	dish A value ±1	dish A value ±1	15	Ready for B002 sample.
if:	Either set (cytotoxicity or % reversal) of A or B codes differ by more than one code				34	Repeat
	Following a TSC = 03 (only possible after achievement of a TSC = 11), a retest will be handled as a dose response. Evaluate exactly as described for a replacement 01 dose response, using the best test result available; however, assign TSC codes as described for a second test (03, 06, or 15).					

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TABLE 2

9ASK TSC ASSIGNMENT
CRUDE MATERIAL TESTING -- B002 SAMPLE (INITIAL TEST)

The B002* sample is associated with confirmation testing and is normally input after a TSC 15 is achieved using a non-BOO2 sample. It will always be input with a TSC of 27 if a natural product. It is required that TSC 27 be associated with a B002* sample number and vice versa. Confirmation testing of a synthetic (no special sample number) will carry a TSC code of 24. Confirmation testing always involves a dose response of at least 3 tests, which are evaluated as a group after examining the individual dose tests within the dose response to confirm that Group A and Group B codes are within a single code of one another. Begin by evaluating each individual dose, and set the TSC codes as follows:

	DISH A		DISH B		COMPUTER ASSIGNMENT	
.	Cytotoxicity Code	% Reversal Code	Cytotoxicity Code	% Reversal Code	TSC	MEANING
if:	≥ 4	any	dish A value ±1	dish A value ±1	05	Cytotoxic
if:	≤ 3	≤ 3	dish A value ±1	dish A value ±1	27N	Noncytotoxic inactive
if:	≤ 3	≥ 4	dish A value ±1	dish A value ±1	27C	Noncytotoxic active
if:	Either set (cytotoxicity or % reversal) of A or B codes differ by more than 1 code:				34	Repeat

After evaluating individual tests, evaluate the total dose response as follows:

(1) If any individual dose has a TSC = 34, entire dose response is coded TSC 34.

(2) If any individual dose is cytotoxic (TSC = 05) and any preceding dose is not, the entire dose response is coded TSC 27A (if Natural Product) -- erratic dose response.

(3) If all individual doses = TSC 05, the dose response is coded TSC = 27T (toxic dose response).**

(4) If none of the above, the TSC for the entire dose response is coded as follows:

Final Dose Response TSC	Condition
27C (complete)	Best individual dose reflects 27C.
27A (repeat)	Best individual dose reflects 27N.

* These rules apply to any numeric B sample equal to or greater than 2.
** TSC 27T dose responses are ignored for purposes of establishing new TSC codes.

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TABLE 3

9ASK TSC ASSIGNMENT
CRUDE MATERIAL TESTING -- B002 SAMPLE (REPEAT TEST)

When a B002* sample is input following a previously processed dose response assigned a 27A, evaluate the individual doses as described below.

	DISH A		DISH B		COMPUTER ASSIGNMENT	
	Cytotoxicity Code	% Reversal Code	Cytotoxicity Code	% Reversal Code		
if	≥ 4	any	dish A value ± 1	dish A value ± 1	05	Cytotoxic
if:	≤ 3	≤ 3	dish A value ± 1	dish A value ± 1	27N	Noncytotoxic inactive
if:	≤ 3	≥ 4	dish A value ± 1	dish A value ± 1	27C	Equivocal
if:	Either set (cytotoxicity or % reversal) of A or B codes differ by more than 1 code:				34	Repeat

Evaluate the overall dose response as follows:

(1) If any individual dose has a TSC = 34, code the entire dose response TSC = 34.

(2) If all individual doses are cytotoxic (TSC = 05), code the entire dose **response** TSC = 27T.** Otherwise, ignore any individual cytotoxic test.

(3) If neither of the above, code the TSC for the entire dose response as follows:

Final Dose Response TSC	Condition
27C	Best individual dose reflects 27C.
27N	Best individual dose does not reflect 27C.

All Natural Product Fractions (sample number begins with D-K or T) are handled

by the computer with no TSC suffix assignment The TSC assigned by the screener

is carried as input.

* These rules apply to any numeric B sample equal to or greater than 2.
** TSC 27T dose responses are ignored for purposes of establishing new TSC codes.

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